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(54) Title: PROCESS FOR IMMOBILIZING ENZYMES TO THE CELL WALL OF A MICROBIAL CELL BY PRODU-CING A FUSION PROTEIN

(57) Abstract

A method is provided for immobilizing an enzyme, comprising immobilizing the enzyme or a functional part thereof to the cell wall of a microbial cell using recombinant DNA techniques. The enzyme is immobilized by linking it to the C-terminal part of a protein that ensures anchoring in the cell wall. Also provided is a recombinant polynucleotide comprising a structural gene encoding an enzyme protein, a part of a gene encoding the C-terminal part of a protein capable of anchoring in a eukaryotic or prokariotic cell wall, as well as a signal sequence, in addition to a chimeric protein encoded by the recombinant polynucleotide and a vector and a microorganism containing the polynucleotide. The microorganism is suitable for carrying out enzymatic pro-cesses on an industrial scale.

Sequence listing and Drawings excluded

PROCESS FOR IMMOBILIZING ENZYMES TO THE CELL WALL OF A MICROBIAL CELL BY PRODUCING A FUSION PROTEIN.

The present invention is in the field of conversion processes using immobilized

5   enzymes, produced by genetic engineering.

Background of the invention

In the detergent, personal care and food products industry there is a strong trend towards natural ingredients of these products and to environmentally acceptable

10   production processes. Enzymic conversions are very important for fulfilling these consumer demands, as these processes can be completely natural. Moreover enzymic processes are very specific and consequently will produce minimum amounts of waste products. Such processes can be carried out in water at mild temperatures and atmospheric pressure. However enzymic processes based on free enzymes are either quite

15   expensive due to the loss of enzymes or require expensive equipment, like ultra-membrane systems to entrap the enzyme.

Alternatively enzymes can be immobilized either physically or chemically. The latter method has often the disadvantage that coupling is carried out using non-natural chemicals and in processes that are not attractive from an environmental point of

20   view. Moreover chemical modification of enzymes is nearly always not very specific, which means that coupling can affect the activity of the enzyme negatively.

Physical immobilization can comply with consumer demands, however also physical immobilization may affect the activity of the enzyme in a negative way. Moreover, a physically immobilized enzyme is in equilibrium with free enzyme, which means that

25   in continuous reactors, according to the laws of thermodynamics, substantial losses of enzyme are unavoidable.

There are a few publications on immobilization of enzymes to microbial cells (see reference 1). The present invention provides a method for immobilizing enzymes to cell walls of microbial cells in a very precise way. Additionally, the immobilization

30   does not require any chemical or physical coupling step and is very efficient.

Some extracellular proteins are known to have special functions which they can perform only if they remain bound to the cell wall of the host cell. Often this type of

protein has a long C-terminal part that anchors it in the cell wall. These C-terminal parts have very special amino acid sequences. A typical example is anchoring via C-terminal sequences enriched in proline (see reference 2). Another mechanism to anchor proteins in cell walls is that the protein has a glycosyl-phosphatidyl-inositol

5   (GPI) anchor (see reference 3) and that the C-terminal part of the protein contains a substantial number of potential serine and threonine glycosylation sites. O-Glycosylation of these sites gives a rod-like conformation to the C-terminal part of these proteins. Another feature of these manno-proteins is that they seem to be linked to the glucan in the cell wall of lower eukaryotes, as they cannot be extracted

10   from the cell wall with SDS, but can be liberated by glucanase treatment.

Summary of the invention

The present invention provides a method for immobilizing an enzyme, which comprises the use of recombinant DNA techniques for producing an enzyme or a

15   functional part thereof linked to the cell wall of a host cell, preferably a microbial cell, and whereby the enzyme or functional fragment thereof is localized at the exterior of the cell wall. Preferably the enzyme or the functional part thereof is immobilized by linking to the C-terminal part of a protein that ensures anchoring in the cell wall.

20   In one embodiment of the invention a recombinant polynucleotide is provided comprising a structural gene encoding a protein providing catalytic activity and at least a part of a gene encoding a protein capable of anchoring in a eukaryotic or prokaryotic cell wall, said part encoding at least the C-terminal part of said anchoring protein. Preferably the polynucleotide further comprises a sequence encoding a signal

25   peptide ensuring secretion of the expression product of the polynucleotide. Such signal peptide can be derived from a glycosyl-phosphatidyl-inositol (GPI) anchoring protein, α-factor, α-agglutinin, invertase or inulinase, α-amylase of *Bacillus*, or a proteinase of lactic acid bacteria. The DNA sequence encoding a protein capable of anchoring in the cell wall can encode α-agglutinin, AGA1, FLO1 or the Major Cell

30   Wall Protein of lower eukaryotes, or a proteinase of lactic acid bacteria. The recombinant polynucleotide is operably linked to a promoter, preferably an inducible

promoter. The DNA sequence encoding a protein providing catalytic activity can
encode a hydrolytic enzyme, e.g. a lipase, or an oxidoreductase, e.g. an oxidase.
Another embodiment of the invention relates to a recombinant vector comprising a
polynucleotide as described above. If such vector contains a DNA sequence encoding
5   a protein providing catalytic activity, which protein exhibits said catalytic activity when
present in a multimeric form, said vector can further comprise a second
polynucleotide comprising a structural gene encoding the same protein providing
catalytic activity combined with a sequence encoding a signal peptide ensuring
secretion of the expression product of said second polynucleotide, said second
10   polynucleotide being operably linked to a regulatable promoter, preferably an
inducible or repressible promoter.
      A further embodiment of the invention relates to a chimeric protein encoded by a
polynucleotide as described above.
      Still another embodiment is a host cell, preferably a microorganism, containing a
15   polynucleotide as described above or a vector as described above. If the protein
providing catalytic activity exhibits said catalytic activity when present in a multimeric
form, said host cell or microorganism can further comprise a second polynucleotide
comprising a structural gene encoding the same protein providing catalytic activity
combined with a sequence encoding a signal peptide ensuring secretion of the
20   expression product of said second polynucleotide, said second polynucleotide being
operably linked to a regulatable promoter, preferably an inducible or repressible
promoter, and said second polynucleotide being present either in another vector or in
the chromosome of said microorganism. Preferably the host cell or microorganism has
at least one of said polynucleotides integrated in its chromosome. As a result of
25   culturing such host cell or microorganism the invention provides a host cell,
preferably a microorganism, having a protein as described above immobilized on its
cell wall. The host cell or microorganism can be a lower eukaryote, in particular a
yeast.
      The invention also provides a process for carrying out an enzymatic process by using
30   an immobilized catalytically active protein, wherein a substrate for said catalytically
active protein is contacted with a host cell or microorganism according to the
invention.

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Brief Description of the Figures

Figure 1: DNA sequence of the 6057 bp HindIII fragment containing the complete AGα1 gene of S. cerevisiae (see SEQ ID NO: 1 and 2). The position of the unique NheI site and the HindIII site used for the described constructions is specified in the
5   header.

Figure 2: Schematic presentation of the construction of pUR2969. The restriction sites for endonucleases used are shown. Abbreviations used: AG-alpha-1: Gene expressing α-agglutinin from S. cerevisiae

amp: ß-lactamase resistance gene
10   PGKp: phosphoglyceratekinase promoter

PGKt: terminator of the same gene.

Figure 3: α-Galactosidase activity of S. cerevisiae MT302/1C cells and culture fluid transformed with pSY13 during batch culture:

A: U/l α-galactosidase per time; the OD_{530} is also shown
15   B: α-galactosidase activity of free and bond enzyme expressed in U/OD_{530}.

Figure 4: α-Galactosidase activity of S. cerevisiae MT302/1C cells and culture fluid transformed with pUR2969 during batch culture:

A: U/l α-galactosidase per time; the OD_{530} is also shown

B: α-galactosidase activity of free and bond enzyme expressed in U/OD_{530}.
20   Figure 5: Western analysis with anti α-galactosidase serum of extracellular fractions of cells of exponential phase (OD_{530}=2). The analyzed fractions are equivalent to 4 mg cell walls, (fresh weight):

A: MT302/1C expressing α-galactosidase,

    lane 1, growth medium
25   lane 2, SDS extract of isolated cell walls

    lane 3, glucanase extract of SDS extracted cell walls;

B: MT302/1C expressing α-Gal-AGα1 fusion protein,

    lane 1, growth medium

    lane 2, SDS extract of isolated cell walls
30   lane 3, glucanase extract of SDS-extracted cell walls

    lane 4: Endo-H treated glucanase extract.

Figure 6: Immunofluorescent labelling (anti α-galactosidase) of MT302/1C cells in the exponential phase (OD$_{530}$=2) expressing the α-Gal-α-agglutinin fusion protein. Phase micrograph of intact cells   A: overview   B: detail.

Figure 7: Schematic presentation of the construction of pUR2970A, pUR2971A, pUR2972A, and pUR2973. The restriction sites for endonucleases used are indicated in the figure. PCR oligonucleotide sequences are mentioned in the text.

Abbreviations used: AGa1 cds: coding sequence of α-agglutinin

a-AGG=AGa1:   Gene expressing α-agglutinin from *S. cerevisiae*

amp: ß-lactamase resistance gene   Pgal7=GAL7:   *GAL7* promoter

lipolase: lipase gene of *Humicola*   invSS: *SUC2* signal sequence

a-MF: prepro-α-mating factor sequence   a-gal: α-galactosidase gene

LEU2d :   truncated promoter of *LEU2* gene;

LEU2 :   *LEU2* gene with complete promoter sequence.

Figure 8: DNA sequence of a fragment containing the complete coding sequence of lipase B of *Geotrichum candidum* strain 335426 (see SEQ ID NO: 11 and 12). The sequence of the mature lipase B starts at nucleotide 97 of the given sequence. The coding sequence starts at nucleotide 40 (ATG).

Figure 9: Schematic presentation of the construction of pUR2975 and pUR2976. The restriction sites for endonucleases used are shown. Abbreviations used:

a-AGG: Gene expressing α-agglutinin from *S. cerevisiae*

amp: ß-lactamase resistance gene   Pgal7=GAL7:   *GAL7* promoter

invSS: *SUC2* signal sequence   a-MF: prepro-α-mating factor sequence

LEU2d: truncated promoter *LEU2* gene   lipolase: lipase gene of *Humicola*

lipaseB: lipaseB gene of *Geotrichum candidum*.

Figure 10: Schematic presentation of the construction of pUR2981 and pUR2982. The restriction sites for endonucleases used are shown. Abbreviations used:

a-AGG=AG-alpha 1:   Gene expressing α-agglutinin from *S. cerevisiae*

mucor lipase: lipase gene of *Rhizomucor miehei*   2u: 2μm sequence

Pgal7=GAL7:   *GAL7* promoter   invSS: *SUC2* signal sequence

a-MF: prepro-α-mating factor sequence   lipolase: lipase gene of *Humicola*

amp: ß-lactamase resistance gene;   LEU2d: truncated promoter *LEU2* gene

LEU2 :   *LEU2* gene with complete promoter sequence.

Figure 11: DNA sequence (2685 bases) of the 894 amino acids coding part of the
FLO1 gene (see SEQ ID NO: 21 and 22), the given sequence starts with the codon
for the first amino acid and ends with the stop codon.

Figure 12: Schematic presentation of plasmid pUR2990. Some restriction sites for en-
5   donucleases relevant for the given cloning procedure are shown.

Figure 13: Schematic presentation of plasmid pUR7034.

Figure 14: Schematic presentation of plasmid pUR2972B.

Figure 15: Immunofluorescent labelling (anti-lipolase) of SU10 cells in the
exponential phase ($OD_{530}=0.5$) expressing the lipolase/-α-agglutinin fusion protein.

10   A: phase micrograph   B: matching fluorescent micrograph

Detailed description of the invention

The present invention provides a method for immobilizing an enzyme, comprising
immobilizing the enzyme or a functional part thereof to the cell wall of a host cell,

15   preferably a microbial cell, using recombinant DNA techniques. In particular, the C-
terminal part of a protein that ensures anchoring in the cell wall is linked to an
enzyme or the functional part of an enzyme, in such a way that the enzyme is
localized on or just above the cell surface. In this way immobilized enzymes are
obtained on the surface of cells. The linkage is performed at gene level and is

20   characterized in that the structural gene coding for the enzyme is coupled to at least
part of a gene encoding an anchor-protein in such a way that in the expression
product the enzyme is coupled at its C-terminal end to the C-terminal part of an
anchor-protein. The chimeric enzyme is preferably preceded by a signal sequence that
ensures efficient secretion of the chimeric protein.

25   Thus the invention relates to a recombinant polynucleotide comprising a structural
gene encoding a protein providing catalytic activity and at least a part of a gene
encoding a protein capable of anchoring in a eukaryotic or prokaryotic cell wall, said
part encoding at least the C-terminal part of said anchoring protein. The length of the
C-terminal part of the anchoring protein may vary. Although the entire structural

30   protein could be used, it is preferred that only a part is used, leading to a more
efficient exposure of the enzyme protein in the medium surrounding the cell. The

anchoring part of the anchoring protein should preferably be entirely present. As an
example, about the C-terminal half of the anchoring protein could be used.
Preferably, the polynucleotide further comprises a sequence encoding a signal peptide
ensuring secretion of the expression product of the polynucleotide. The signal peptide

5   can be derived from a GPI anchoring protein, α-factor, α-agglutinin, invertase or
inulinase, α-amylase of *Bacillus*, or a proteinase of lactic acid bacteria.
The protein capable of anchoring in the cell wall is preferably selected form the
group of α-agglutinin, AGA1, FLO1 (flocculation protein) or the Major Cell Wall
Protein of lower eukaryotes, or a proteinase of lactic acid bacteria. The

10 polynucleotide of the invention is preferably operably linked to a promoter, preferably
a regulatable promoter, especially an inducible promoter.
The invention also relates to a recombinant vector containing the polynucleotide as
described above, and to a host cell containing this polynucleotide, or this vector.
In a particular case, wherein the protein providing catalytic activity exhibits said

15 catalytic activity when present in a multimeric form, such as may be the case with
oxidoreductases, dimerisation or multimerisation of the monomers might be a
prerequisite for activity. The vector and/or the host cell can then further comprise a
second polynucleotide comprising a structural gene encoding the same protein pro-
viding catalytic activity combined with a sequence encoding a signal peptide ensuring

20 secretion of the expression product of said second polynucleotide, said second
polynucleotide being operably linked to a regulatable promoter, preferably an
inducible or repressible promoter. Expression and secretion of the second
polynucleotide after expression and secretion of the first polynucleotide will then
result in the formation of an active multimer on the exterior of the cell wall.

25 The host cell or microorganism preferably contains the polynucleotide described
above, or at least one of said polynucleotides in the case of a combination, integrated
in its chromosome.
The present invention relates in particular to lower eukaryotes like yeasts that have
very stable cell walls and have proteins that are known to be anchored in the cell

30 wall, e.g. α-agglutinin or the product of gene *FLO1*. Suitable yeasts belong to the
genera *Candida, Debaryomyces, Hansenula, Kluyveromyces, Pichia* and *Saccharomyces*.

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Also fungi, especially *Aspergillus, Penicillium* and *Rhizopus* can be used. For certain applications also prokaryotes are applicable.

For yeasts the present invention deals in particular with genes encoding chimeric enzymes consisting of:

5 a.   the signal sequence e.g. derived from the α-factor-, the invertase-, the α-agglutinin- or the inulinase genes;

   b.   structural genes encoding hydrolytic enzymes such as α-galactosidase, proteases, peptidases, pectinases, pectylesterase, rhamnogalacturonase, esterases and lipases, or non-hydrolytic enzymes such as oxidases; and

10 c.   the C-terminus of typically cell wall bound proteins such as α-agglutinin (see reference 4), AGA1 (see reference 5) and FLO1 (see the non-prior published reference 6).

The expression of these genes can be under the control of a constitutive promoter, but more preferred are regulatable, i.e. repressible or inducible promoters such as the

15 *GAL7* promoter for *Saccharomyces*, or the inulinase promoter for *Kluyveromyces* or the methanol-oxidase promoter for *Hansenula*.

Preferably the constructs are made in such a way that the new genetic information is integrated in a stable way in the chromosome of the host cell.

The invention further relates to a host cell, in particular a microorganism, having the

20 chimeric protein described above immobilized on its cell wall. It further concerns the use of such microorganisms for carrying out an enzymatic process by contacting a substrate for the enzyme with the microorganism. Such a process may be carried out e.g. in a packed column, wherein the microorganisms may be supported on solid particles, or in a stirred reactor. The reaction may be aqueous or non-aqueous. Where

25 necessary, additives necessary for the performance of the enzyme, e.g. a co-factor, may be introduced in the reaction medium.

After repeated usage of the naturally immobilized enzyme system in processes, the performance of the system may decrease. This is caused either by physical denaturation or by chemical poisoning or detachment of the enzyme. A particular

30 feature of the present invention is that after usage the system can be recovered from the reaction medium by simple centrifugation or membrane filtration techniques and that the thus collected cells can be transferred to a recovery medium in which the

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cells revive quickly and concomitantly produce the chimeric protein, thus ensuring that the surface of the cells will be covered by fully active immobilized enzyme. This regeneration process is simple and cheap and therefore will improve the economics of enzymic processes and may result in a much wider application of processes based on

5 immobilized enzyme systems.

However, by no means the present invention is restricted to the reusability of the immobilized enzymes.

The invention will be illustrated by the following examples without the scope of the invention being limited thereto.

10

EXAMPLE 1   **Immobilized α-galactosidase/α-agglutinin on the surface of S. cerevisiae.**

The gene encoding α-agglutinin has been described by Lipke *et al.* (see reference 4). The sequence of a 6057 bp *Hind*III insert in pTZ18R, containing the whole AGα1

15 gene is given in Figure 1. The coding sequence expands over 650 amino acids, including a putative signal sequence starting at nucleotide 3653 with ATG. The unique *Nhe*I site cuts the DNA at position 988 of the given coding sequence within the coding part of amino acid 330, thereby separating the α-agglutinin into an N-terminal and a C-terminal part of about same size.

20 Through digestion of pUR2968 (see Figure 2) with *Nhe*I/*Hind*III a 1.4 kb fragment was released, containing the sequence information of the putative cell wall anchor. For the fusion to α-galactosidase the plasmid pSY16 was used, an episomal vector based on YEplac 181, harbouring the α-galactosidase sequence preceded by the *SUC2* invertase signal sequence and placed between the constitutive *PGK* promoter and

25 *PGK* terminator. The *Sty*I site, present in the last nine base-pairs of the open reading frame of the α-galactosidase gene, was ligated to the *Nhe*I site of the *AGα1* gene fragment. To ensure the in frame fusion, the *Sty*I site was filled in and the 5' overhang of the *Nhe*I site was removed, prior to ligation into the *Sty*I/ *Hind*III digested pSY13 (see Figure 2).

30 To verify the correct assembly of the new plasmid, the shuttle vector was transformed into *E. coli* JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F' [*traD36 proAB+ lacI*q *lacZ*ΔM15]) (see reference 7) by the transformation protocol

described by Chung *et al.* (see reference 8). One of the positive clones, designated pUR2969, was further characterized, the DNA isolated and purified according to the Quiagen protocol and subsequently characterized by DNA sequencing. DNA sequencing was mainly performed as described by Sanger *et al.* (see reference 9), and

5   Hsiao (see reference 10), here with the Sequenase version 2.0 kit from United States Biochemical Company, according to the protocol with T7 DNA polymerase (Amersham International plc) and [^{35}S]dATPαS (Amersham International plc: 370 MBq/ml; 22 TBq/mmol).

This plasmid was then transformed into *S. cerevisiae* strain MT302/1C according to

10   the protocol from Klebe *et al.* (see reference 11).

Yeast transformants were selected on selective plates, lacking leucine, on with 40 µl (20mg/ml DMF). X-α-Gal (5-bromo-4-chloro-3-indolyl-α-D-glucose, Boehringer Mannheim) was spread, to directly test for α-galactosidase activity (see reference 12). To demonstrate the expression, secretion, localization and activity of the chimeric

15   protein the following analyses were performed:

<u>1. Expression and secretion</u>

S. cerevisiae strain MT302/1C was transformed with either plasmid pSY13 containing the α-galactosidase gene of *Cyamopsis tetragonoloba* or plasmid pUR2969 containing the α-galactosidase/α-agglutinin fusion construct. During batch culture α-galactosidase

20   activities were determined for washed cells and growth medium. The results are given in Figure 3 and Figure 4. The α-galactosidase expressed from yeast cells containing plasmid pSY13 was almost exclusively present in the growth medium (Figure 3A), whereas the α-galactosidase-α-agglutinin fusion protein was almost exclusively cell associated (Figure 4A). Moreover, the immobilized, cell wall-associated, α-galacto-

25   sidase-α-agglutinin fusion enzyme had retained the complete activity over the whole incubation time, while the secreted and released enzyme lost about 90% of the activity after an incubation of 65 hours. This indicates, that the immobilization of the described enzyme into the cell wall of yeast protects the enzyme against inactivation, presumably through proteinases, and thereby increases the stability significantly.

30   Further insight into the localization of the different gene products was obtained by Western analysis. Therefore, cells were harvested by centrifugation and washed in 10 mM Tris.HCl, pH 7.8; 1 mM PMSF at 0°C and all subsequent steps were performed

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at the same temperature. Three ml isolation buffer and 10 g of glass beads were added per gram of cells (wet weight). The mixture was shaken in a Griffin shaker at 50% of its maximum speed for 30 minutes. The supernatant was isolated and the glass beads were washed with 1 M NaCl and 1 mM PMSF until the washes were

5   clear. The supernatant and the washes were pooled. The cell walls were recovered by centrifugation and were subsequently washed in 1 mM PMSF.
Non-covalently bound proteins or proteins bound through disulphide bridges were released from cell walls by boiling for 5 minutes in 50 mM Tris.HCl, pH 7.8; containing 2 % SDS, 100 mM EDTA and 40 mM ß-mercaptoethanol. The SDS-

10   extracted cell walls were washed several times in 1 mM PMSF to remove SDS. Ten mg of cell walls (wet weight) were taken up in 20 l 100 mM sodium acetate, pH 5.0, containing 1 mM PMSF. To this, 0.5 mU of the ß-1,3-glucanase (Laminarase; Sigma L5144) was used as a source of ß-1,3-glucanase) was added followed by incubation for 2 hours at 37 °C. Subsequently another 0.5 mU of ß-1,3-glucanase was added,

15   followed by incubation for another 2 hours at 37 °C.
Proteins were denatured by boiling for 5 minutes preceding Endo-H treatment. Two mg of protein were incubated in 1 ml 50 mM potassium phosphate, pH 5.5, containing 100 mM ß-mercaptoethanol and 0.5 mM PMSF with 40 mU Endo-H (Boehringer) for 48 hours at 37 °C. Subsequently 20 mU Endo-H were added

20   followed by 24 hours of incubation at 37 °C.
Proteins were separated by SDS-PAGE according to Laemmli (see reference 13) in 2.2.-20% gradient gels. The gels were blotted by electrophoretic transfer onto Immobilon polyvinylidene-difluoride membrane (Millipore) as described by Towbin *et al.* (see reference 14). In case of highly glycosylated proteins a subsequently mild

25   periodate treatment was performed in 50 mM periodic acid, 100 mM sodium acetate, pH 4.5, for several hours at 4 °C. All subsequent incubations were carried out at room temperature. The blot was blocked in PBS, containing 0.5% gelatine and 0.5% Tween-20, for one hour followed by incubation for 1 hour in probe buffer (PBS, 0.2% gelatine, 0.1% Tween-20) containing 1:200 diluted serum. The blot was subsequently

30   washed several times in washing buffer (PBS; 0.2% gelatine; 0.5% Tween-20) followed by incubation for 1 hour in probe-buffer containing ^{125}I-labelled protein A

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(Amersham). After several washes in washing buffer, the blot was air-dried, wrapped
in Saran (Dow) and exposed to X-omat S film (Kodak) with intensifying screen at
-70 °C. An Omnimedia 6cx scanner and the Adobe Photoshop programme were used
to quantify the amount of labelled protein. The results of the various protein isolation

5 procedures from both transformants are given in Figure 5. While for the
transformants comprising the pSY13 plasmid the overall mass of the enzyme was
localized in the medium, with only minor amounts of enzyme more entrapped than
bond in the cell wall (Figure 5A) -which could completely be removed by SDS extrac-
tion- the fusion protein was tightly bound to the cell wall; with only small amounts of

10 α-galactosidase/α-agglutinin delivered into the surrounding culture fluid or being SDS
extractable. In contrast to the laminarinase extraction of cell walls from cells
expressing the free α-galactosidase, where no further liberation of any more enzyme
was observed, identical treatment of fusion enzyme expressing cells released the
overall bulk of the enzyme. This indicates that the fusion protein is intimately

15 associated with the cell wall glucan in S. cerevisiae, like α-agglutinin, while α-galactosi-
dase alone is not. The subsequently performed EndoH treatment showed a heavy
glycosylation of the fusion protein, a result, entirely in agreement with the described
extended glycosylation of the C-terminal part of α-agglutinin.

2. Localization

20 Immunofluorescent labelling with anti-α-galactosidase serum was performed on intact
cells to determine the presence and distribution of α-galactosidase/α-agglutinin fusion
protein in the cell wall. Immunofluorescent labelling was carried out without fixing
according to Watzele et al. (see reference 15). Cells of $OD_{530}=2$ were isolated and
washed in TBS (10 mM Tris.HCl, pH 7.8, containing 140 mM NaCl, 5 mM EDTA

25 and 20 μg/ml cycloheximide). The cells were incubated in TBS + anti-α-galactosidase
serum for 1 hour, followed by several washings in TBS. A subsequent incubation was
carried out with FITC-conjugated anti-rabbit IgG (Sigma) for 30 minutes. After
washing in TBS, cells were taken up in 10 mM Tris.HCl, pH 9.0, containing 1 mg/ml
p-phenylenediamine and 0.1 % azide and were photographed on a Zeiss 68000

30 microscope. The results of these analysis are given in Figure 6, showing clearly that
the chimeric α-galactosidase/α-agglutinin is localized at the surface of the yeast cell.
Buds of various sizes, even very small ones very uniformly labelled, demonstrates that

the fusion enzyme is continuously incorporated into the cell wall throughout the cell cycle and that it instantly becomes tightly linked.

3. Activity

To quantitatively assay α-galactosidase activity, 200 μl samples containing 0.1 M

5   sodium-acetate, pH 4.5 and 10 mM p-nitrophenyl-α-D-galactopyranoside (Sigma) were incubated at 37 °C for exactly 5 minutes. The reaction was stopped by addition of 1 ml 2% sodium carbonate. From intact cells and cell walls, removed by centrifugation and isolated and washed as described, the α-galactosidase activity was calculated using the extinction coefficient of p-nitrophenol of 18.4 cm^2/mole at 410 nm.

10   One unit was defined as the hydrolysis of 1 μmole substrate per minute at 37 °C.

Table 1. Distribution of free and immobilized α-galactosidase activity in yeast cells

Expressed protein	α-Galactosidase activity (U/g F.W. cells)		
	Growth medium	Intact cells	Isolated cell walls
α-galactosidase	14.7	0.37	0.01
αGal/αAGG fusion protein	0.54	13.3	10.9

20   Transformed MT302/1C cells were in exponential phase (OD_{530}=2). One unit is defined as the hydrolysis of 1 μmole of p-nitrophenyl-α-D-galactopyranoside per minute at 37 °C.

25   The results are summarized in Table 1. While the overall majority of α-galactosidase was distributed in the culture fluid, most of the fusion product was associated with the cells, primarily with the cell wall. Taking together the results shown in Figures 3 to 6 and in Table 1, it could be calculated that the enzymatic α-galactosidase activity of the chimeric enzyme is as good as that of the free enzyme. Moreover, during

30   stationary phase, the activity of the α-galactosidase in the growth medium decreased, whereas the activity of the cell wall associated α-galactosidase α-agglutinin fusion

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remained constant, indicating that the cell associated fusion protein is protected from inactivation or proteolytic degradation.

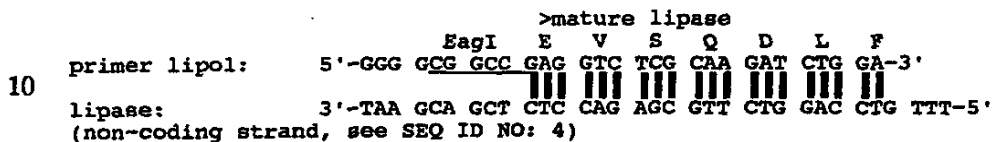N.B. The essence of this EXAMPLE was published during the priority year by M.P.

5   Schreuder *et al.* (see reference 25).

EXAMPLE 2A   **Immobilized *Humicola* lipase/α-agglutinin on the surface of *S. cerevisiae.* (inducible expression of immobilized enzyme system)**
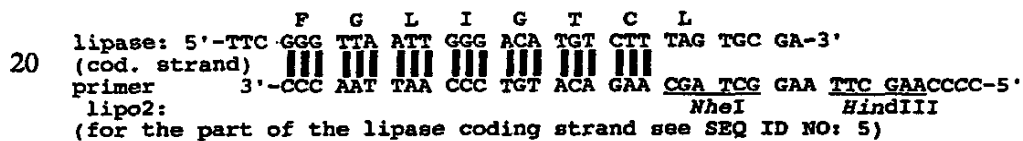
The construction and isolation of the 1.4 kb *Nhe*I/*Hind*III fragment containing the C-

10   terminal part of α-agglutinin has been described in EXAMPLE 1. Plasmid pUR7021 contains an 894 bp long synthetically produced DNA fragment encoding the lipase of *Humicola* (see reference 16 and SEQ ID NO: 7 and 8), cloned into the *Eco*RI/*Hind*III restriction sites of the commercially available vector pTZ18R (see Figure 7). For the proper one-step modification of both the 5' end and the 3' end of

15   the DNA part coding for the mature lipase, the PCR technique can be applied. Therefore the DNA oligonucleotides lipo1 (see SEQ ID NO: 3) and lipo2 (see SEQ ID NO: 6) can be used as primers in a standard PCR protocol, generating an 826 bp long DNA fragment with an *Eag*I and a *Hind*III restriction site at the ends, which can be combined with the larger part of the *Eag*I/*Hind*III digested pUR2650, a plasmid

20   containing the α-galactosidase gene preceded by the invertase signal sequence as described earlier in this specification, thereby generating plasmid pUR2970A (see Figure 7).

PCR oligonucleotides for the in-frame linkage of *Humicola* lipase and the C-terminus of α agglutinin.

a: PCR oligonucleotides for the transition between *SUC2* signal sequence and the N-terminus of lipase.

```
                                    >mature lipase
                              EagI    E   V   S   Q   D   L   F
primer lipo1:      5'-GGG GCG GCC GAG GTC TCG CAA GAT CTG GA-3'
                              |||  ||| ||| ||| ||| ||| ||
lipase:            3'-TAA GCA GCT CTC CAG AGC GTT CTG GAC CTG TTT-5'
(non-coding strand, see SEQ ID NO: 4)
```

b: PCR oligonucleotides for the in frame transition between C-terminus of lipase and C-terminal part of α-agglutinin.

```
                    F   G   L   I   G   T   C   L
lipase: 5'-TTC GGG TTA ATT GGG ACA TGT CTT TAG TGC GA-3'
(cod. strand)    |||  ||| ||| ||| ||| ||| |||
primer      3'-CCC AAT TAA CCC TGT ACA GAA CGA TCG GAA TTC GAACCCC-5'
 lipo2:                                      NheI        HindIII
(for the part of the lipase coding strand see SEQ ID NO: 5)
```

Through the PCR method a *Nhe*I site will be created at the end of the coding sequence of the lipase, allowing the in-frame linkage between the DNA coding for lipase and the DNA coding for the C-terminal part of α-agglutinin. Plasmid pUR2970A can then be digested with *Nhe*I and *Hind*III and the 1.4 kb *Nhe*I/*Hind*III fragment containing the C-terminal part of α-agglutinin from plasmid pUR2968 can be combined with the larger part of *Nhe*I and *Hind*III treated plasmid pUR2970A, resulting in plasmid pUR2971A. From this plasmid the 2.2 kb *Eag*I/*Hind*III fragment can be isolated and ligated into the *Eag*I- and *Hind*III-treated pUR2741, whereby plasmid pUR2741 is a derivative of pUR2740 (see reference 17), where the second *Eag*I restriction site in the already inactive *Tet* resistance gene was deleted through *Nru*I/*Sal*I digestion. The *Sal*I site was filled in prior to religation. The ligation then results in pUR2972A containing the *GAL7* promoter, the invertase signal sequence, the chimeric lipase/α-agglutinin gene, the 2 μm sequence, the defective *Leu2* promoter and the *Leu2* gene. This plasmid can be used for transforming *S. cerevisiae* and the transformed cells can be cultivated in YP medium containing galactose as an inducer without repressing amounts of glucose being present, which causes the expression of the chimeric lipase/α-agglutinin gene.

The expression, secretion, localization and activity of the chimeric lipase/α-agglutinin can be analyzed using similar procedures as given in EXAMPLE 1.

In a similar way variants of *Humicola* lipase, obtained via rDNA techniques, can be
5   linked to the C-terminal part of α-agglutinin, which variants can have a higher stability during (inter)esterification processes.

EXAMPLE 2B   Immobilized *Humicola* lipase/α-agglutinin on the surface of *S. cerevisiae* (inducible expression of immobilized enzyme system)

10 EXAMPLE 2A describes a protocol for preparing a particular construct. Before carrying out the work it was considered more convenient to use the expression vector described in EXAMPLE 1, so that the construction route given in this EXAMPLE 2B differs on minor points from the construction route given in EXAMPLE 2A and the resulting plasmids are not identical to those described in EXAMPLE 2A. However,
15 the essential gene construct comprising the promoter, signal sequence, and the structural gene encoding the fusion protein are the same in EXAMPLES 2A and 2B.

1.   Construction

The construction and isolation of the 1.4 kb *Nhe*I/*Hind*III fragment encoding the C-
20 terminal part of α-agglutinin cell wall protein has been described in EXAMPLE 1. The plasmid pUR7033 (resembling pUR7021 of EXAMPLE 2A) was made by treating the commercially available vector pTZ18R with *Eco*RI and *Hind*III and ligating the resulting vector fragment with an 894 bp long synthetically produced DNA *Eco*RI/*Hind*III fragment encoding the lipase of *Humicola* (see SEQ ID NO: 7
25 and 8, and reference 16).

For the fusion of the lipase to the C-terminal, cell wall anchor-comprising domain of α-agglutinin, plasmid pUR7033 was digested with *Eag*I and *Hind*III, and the lipase coding sequence was isolated and ligated into the *Eag*I- and *Hind*III-digested yeast expression vector pSY1 (see reference 27), thereby generating pUR7034 (see Figure
30 13). This is a 2μm episomal expression vector, containing the α-galactosidase gene described in EXAMPLE 1, preceded by the invertase (*SUC2*) signal sequence under the control of the inducible *GAL7* promoter.

Parallel to this digestion, pUR7033 was also digested with *Eco*RV and *Hind*III,
thereby releasing a 57 bp long DNA fragment, possessing codons for the last 15 car-
boxyterminal amino acids. This fragment was exchanged against a small DNA frag-
ment, generated through the hybridisation of the two chemically synthesized

5   deoxyoligonucleotides SEQ ID NO: 9 and SEQ ID NO: 10. After annealing of both
DNA strands, these two oligonucleotides essentially reconstruct the rest of the 3'
coding sequence of the initial lipase gene, but additionally introduce downstream of
the lipase gene a new *Nhe*I restriction site, followed by a *Hind*III site in close vicinity,
whereby the first three nucleotides of the *Nhe*I site form the codon for the last amino

10   acid of the lipase. The resulting plasmid was designated pUR2970B. Subsequently,
this construction intermediate was digested with *Eag*I and *Nhe*I, the lipase encoding
fragment was isolated, and, together with the 1.4 kb *Nhe*I/*Hind*III fragment of
pUR2968 ligated into the *Eag*I- and *Hind*III-cut pSY1 vector. The outcome of this 3-
point-ligation was called pUR2972B (see Figure 14), the final lipolase-α-agglutinin

15   yeast expression vector.

This plasmid was used for transforming *S. cerevisiae* strain SU10 as described in
reference 17 and the transformed cells were cultivated in YP medium containing
galactose as the inducer without repressing amounts of glucose being present, which
causes the expression of the chimeric lipase/α-agglutinin gene.

20   2.   Activity

To quantify the lipase activity, two activity measurements with two separate substrates
were performed. In both cases, SU10 yeast cells transformed with either plasmid
pUR7034 or pSY1 served as control. Therefore, yeast cell transformants containing
either plasmid pSY1 or plasmid pUR7034 or plasmid pUR2972B were grown up for

25   24h in YNB-glucose medium supplied with histidine and uracil, then diluted 1:10 in
YP-medium supplied with 5% galactose, and again cultured. After 24h incubation at
30°C, a first measurement for both assays was performed.

The first assay applied was the pH stat method. Within this assay, one unit of lipase
activity is defined as the amount of enzyme capable of liberating one micromole of

30   fatty acid per minute from a triglyceride substrate under standard assay conditions (30
ml assay solution containing 38 mM olive oil, considered as pure trioleate, emulsified
with 1:1 w/w gum arabic, 20 mM calcium chloride, 40 mM sodium chloride, 5 mM

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Tris, pH 9.0, 30°C) in a radiometer pH stat apparatus (pHM 84 pH meter, ABU 80 autoburette, TTA 60 titration assembly). The fatty acids formed were titrated with 0.05 N NaOH and the activity measured was based on alkali consumption in the interval between 1 and 2 minutes after addition of putative enzyme batch. To test for
5   immobilized lipase activity, 1 ml of each culture was centrifuged, the supernatant was saved, the pellet was resuspended and washed in 1 ml 1 M sorbitol, subsequently again centrifuged and resuspended in 200μl 1 M sorbitol. From each type of yeast cell the first supernatant and the washed cells were tested for lipase activity.

10   A: Lipase activity after 24h (LU/ml)

	cell bound	culture fluid
pSY1	5.9	8.8
pUR7034	24.1	632.0
pUR2972B-(1)	18.7	59.6
pUR2972B-(2)	24.6	40.5

B: Lipase activity after 48h (LU/ml)

	cell bound	culture fluid	OD660
pSY1	6.4	4.3	¯40
pUR7034	215.0	2750.0	¯40
pUR2972B-(1)	37.O	87.0	¯40
pUR2972B-(2)	34.0	82.0	¯40

The rest of the yeast cultures was further incubated, and essentially the same
25   separation procedure was done after 48 hours. Dependent on the initial activity measured, the actual volume of the sample measured deviated between 25μl and 150μl.
This series of measurements indicates, that yeast cells comprising the plasmid coding for the lipase-α-agglutinin fusion protein in fact express some lipase activity which is
30   associated with the yeast cell.

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An additional second assay was performed to further confirm the immobilization of activity of lipase on the yeast cell surface. Briefly, within this assay, the kinetics of the PNP (=paranitrophenyl) release from PNP-butyrate is determined by measurement of the OD at 400 nm. Therefore, 10 ml cultures containing yeast cells with either pSY1,

5 pUR7034 or pUR2972B were centrifuged, the pellet was resuspended in 4 ml of buffer A (0.1 M NaOAc, pH 5.0 and 1 mM PMSF), from this 4 ml 500µl was centrifuged again and resuspended in 500 µl PNB-buffer (20 mM Tris-HCl, pH 9.0, 20 mM CaCl2, 25 mM NaCl), centrifuged once again, and finally resuspended in 400µl PNB buffer. This fraction was used to determine the cell bound fraction of

10 lipase.

The remaining 3500µl were spun down, the pellet was resuspended in 4 ml A, to each of this, 40µl laminarinase (ex mollusc, 1.25 mU/µl) was added and first incubated for 3 hours at 37°C, followed by an overnight incubation at 20°C. Then the reaction mixture, still containing intact cells, were centrifuged again and the supernatant was

15 used to determined the amount of originally cell wall bound material released through laminarinase incubation. The final pellet was resuspended in 400µl PNP buffer, to calculate the still cell associated part. The blank reaction of a defined volume of specific culture fraction in 4 ml assay buffer was determined, and than the reaction was started through addition of 80µl of substrate solution (100 mM PNP-

20 butyrate in methanol), and the reaction was observed at 25°C at 400 nm in a spectrophotometer.

	cell bound activity*	activity in the medium	laminarinase extract	laminarinase extracted cells	OD660
25 pSY1	0.001 (116µl)	0.001	0.028	0.000	2.6
pUR7034	0.293 (220µl)	0.446	0.076	0.985	2.36
pUR2972B-(1)	0.494 (143µl)	0.021	0.170	0.208	2.10

* unless otherwise mentioned, the volume of enzyme solution added was 20µl

30

This result positively demonstrates that a significant amount of lipase activity is immobilized on the surface yeast cell, containing plasmid pUR2972B. Here again,

incorporation took place in such a way, that the reaction was catalyzed by cell wall
inserted lipase of intact cells, indicated into the exterior orientated immobilization.
Furthermore, the release of a significant amount of lipase activity after incubation
with laminarinase again demonstrates the presumably covalent incorporation of a
5   heterologous enzyme through gene fusion with the C-terminal part of α-agglutinin.

3.   Localization

The expression, secretion, and subsequent incorporation of the lipase-α-agglutinin
fusion protein into the yeast cell wall was also confirmed through immunofluorescent
labelling with anti-lipolase serum essentially as described in EXAMPLE 1, item
10   2. Localization.

As can be seen in Figure 15, the immunofluorescent stain shows essentially an
analogous picture as the α-galactosidase immuno stain, with clearly detectable
reactivity on the outside of the cell surface (see Figure 15 A showing a clear halo
around the cells and Figure B showing a lighter circle at the surface of the cells), but
15   neither in the medium nor in the interior of the cells. Yeast cells expressing
pUR2972B, the *Humicola* lipase-α-agglutinin fusion protein, become homogeneously
stained on the surface, indicating the virtually entire immobilization of a chimeric
enzyme with an α-agglutinin C-terminus on the exterior of a yeast cell. In the
performed control experiment SU10 yeast cells containing plasmid pUR7034 served
20   as a control and here, no cell surface bound reactivity against the applied anti-lipase
serum could be detected.

In a similar way variants of *Humicola* lipase, obtained via rDNA techniques, can be
linked to the C-terminal part of α-agglutinin, which variants can have a higher
stability during (inter)esterification processes.

25

EXAMPLE 3   Immobilized *Humicola* lipase/α-agglutinin on the surface of *S.*
                 ***cerevisiae* (constitutive expression of immobilized enzyme system)**

Plasmid pUR2972 as described in EXAMPLE 2 can be treated with *Eag*I and *Hind*III
and the about 2.2 kb fragment containing the lipase/α-agglutinin gene can be
30   isolated. Plasmid pSY16 can be restricted with *Eag*I and *Hind*III and between these
sites the 2.2 kb fragment containing the lipase/α-agglutinin fragment can be ligated
resulting in pUR2973. The part of this plasmid that is involved in the production of

the chimeric enzyme is similar to pUR2972 with the exception of the signal sequence.
Whereas pUR2972 contains the *SUC2*-invertase-signal sequence, .pUR2973 contains
the α-mating factor signal sequence (see reference 18). Moreover the plasmid
pUR2973 contains the *Leu2* marker gene with the complete promoter sequence,
5   instead of the truncated promoter version of pUR2972.

EXAMPLE 4   Immobilized *Geotrichum* lipase/α-agglutinin on the surface of *S.*
                        cerevisiae

The construction and isolation of the 1.4 kb *NheI/HindIII* fragment comprising the
10   C-terminal part of AGα-1 (α-agglutinin) gene has been described in EXAMPLE 1.
For the in-frame gene fusion of the DNA coding for the C-terminal membrane
anchor of α-agglutinin to the complete coding sequence of *Geotrichum candidum*
lipase B from strain CMICC 335426 (see Figure 8 and SEQ ID NO: 11 and 12), the
plasmid pUR2974 can be used. This plasmid, derived from the commercially available
15   pBluescript II SK plasmid, contains the cDNA coding for the complete *G. candidum*
lipase II on an 1850 bp long *EcoRI/XhoI* insert (see Figure 9).
To develop an expression vector for *S. cerevisiae* with homologous signal sequences,
the N-terminus of the mature lipase B was determined experimentally by standard
techniques. The obtained amino acid sequence of "Gln-Ala-Pro-Thr-Ala-Val..." is in
20   complete agreement with the cleavage site of the signal peptidase on the *G. candidum*
lipase II (see reference 19).
For the fusion of the mature lipase B to the *S. cerevisiae* signal sequences of *SUC2*
(invertase) or α-mating factor (prepro-αMF) on one hand and the in-frame fusion to
the 3' part of the AGα1 gene PCR technique can be used. The PCR primer lipo3
25   (see SEQ ID NO: 13) can be constructed in such a way, that the originally present
*Eag*I site in the 5'-part of the coding sequence (spanning codons 5-7 of the mature
protein) will become inactivated without any alteration in the amino acid sequence.
To facilitate the subsequent cloning procedures, the PCR primer can further contain
a new *Eag*I site at the 5' end, for the in-frame ligation to *SUC2* signal sequence or
30   prepro-αMF sequence, respectively. The corresponding PCR primer lipo4 (see SEQ
ID NO: 16) contains an extra *Nhe*I site behind the nucleotides coding for the

22

C-terminus of lipase B, to ensure the proper fusion to the C-terminal part of
α-agglutinin.

PCR oligonucleotides for the in frame linkage of *G. candidum* lipase II to the
SUC2 signal sequence and the C-terminal part of α-agglutinin.

a: N-terminal transition to either prepro αMF sequence or *SUC2* signal sequence.

```
                   EagI    A    Q    A    P    R    P    S    L    N
primer lipo3:  5'-GGG GCG GCC GCG CAG GCC CCA AGG CGG TCT CTC AAT-3'
                        ‖‖  ‖‖‖ ‖‖‖ ‖‖‖ ‖‖ ‖‖‖ ‖‖‖ ‖‖‖ ‖‖‖
lipaseII:              3'-GAC CGG GTC CGG GGT GCC GCC AGA GAG TTA-5'
(non-cod. strand, see SEQ ID NO: 14) )
```


b: C-terminal fusion to C part of α-agglutinin

```
            S    N    F    E    T    D    V    N    L    Y    G
lipase:  5'-CA AAC TTT GAG ACT GAC GTT AAT CTC TAC GGT TAA AAC-3'
(cod. strand)        ‖ ‖‖‖ ‖‖‖ ‖‖‖ ‖‖‖ ‖‖‖ ‖‖‖ ‖‖‖
primer lipo4:       3'-C TGA CTG CAA TTA GAG ATG CCA CGATCG CCCC-5'
                                                        NheI
(for the part of the lipase coding strand see SEQ ID NO: 15)
```

The PCR product with the modified ends can be generated by standard PCR
protocols, using instead of the normal Ampli-*Taq* polymerase the new thermostable
VENT polymerase, which also exhibits proofreading activity, to ensure an error-free
DNA template. Through digestion of the formerly described plasmid pUR2972 with
*Eag*I (complete) and *Nhe*I (partial), the *Humicola* lipase fragment can be exchanged
against the DNA fragment coding for lipase B, thereby generating the final *S.
cerevisiae* expression vector pUR2975 (see Figure 9).

The *Humicola* lipase-α-agglutinin fusion protein coding sequence can be exchanged
against the lipase B/α-agglutinin fusion construct described above by digestion of the
described vector pUR2973 with *Eag*I/*Hind*III, resulting in pUR2976 (see Figure 9).

EXAMPLE 5   Immobilized *Rhizomucor miehei* lipase/α-agglutinin on the surface
                 of *S. cerevisiae*

The construction and isolation of the 1.4 kb *Nhe*I/*Hind*III fragment encoding the
C-terminal part of α-agglutinin has been described in EXAMPLE 1. The plasmid
pUR2980 contains a 1.25 kb cDNA fragment cloned into the *Sma*I site of
commercially available pUC18, which (synthetically synthesizable) fragment encodes

the complete coding sequence of triglyceride lipase of *Rhizomucor miehei* (see
reference 20), an enzyme used in a number of processes to interesterify
triacylglycerols (see reference 21) or to prepare biosurfactants (see reference 22).
Beside the 269 codons of the mature lipase molecule, the fragment also harbours

5   codons for the 24 amino acid signal peptide as well as 70 amino acids of the
propeptide. PCR can easily be applied to ensure the proper fusion of the gene frag-
ment encoding the mature lipase to the *SUC2* signal sequence or the prepro α-mating
factor sequence of *S. cerevisiae*, as well as the in-frame fusion to the described
*Nhe*I/*Hind*III fragment. The following two primers, lipo5 (see SEQ ID NO: 17) and

10 lipo6 (see SEQ ID NO: 20), will generate a 833 bp DNA fragment, which after
Proteinase K treatment and digestion with *Eag*I and *Nhe*I can be cloned as an 816 bp
long fragment into the *Eag*I/*Nhe*I digested plasmids pUR2972 and pUR2973,
respectively (see Figure 7).

```
                        EagI    A    S    I    D    G    G    I
15  lipo5:        5'-CCC GCG GCC GCG AGC ATT GAT GGT GGT ATC-3'
                          III  III  III  III  III  III
    lipase (non-cod. strand):   3'-TCG TAA CTA GCA CCA TAG-5'
    (for the part of the lipase non-coding strand see SEQ ID NO: 18)

                        N    T    G    L    C    T
    lipase (cod. strand):   5'-AAC ACA GGC CTC TGT ACT-3'
                                III  III  III  III  III  III
    Lipo6:              3'-TTG TGT CCG GAG ACA TGA CGATCGCGCC-5'
                                                        NheI
25  (for the part of the lipase coding strand see SEQ ID NO: 19)
```

These new *S. cerevisiae* expression plasmids contain the *GAL7* promoter, the invertase
signal sequence (pUR2981) or the prepro-α-mating factor sequence (pUR2982), the
chimeric *Rhizomucor miehei* lipase/α-agglutinin gene, the 2 μm sequence, the

30 defective (truncated) *Leu2* promoter and the *Leu2* gene. These plasmids can be
transformed into *S. cerevisiae* and grown and analyzed using protocols described in
earlier EXAMPLES.

EXAMPLE 6   Immobilized *Aspergillus niger* glucose oxidase/GPI anchored cell
35   wall proteins on the surface of *S. cerevisiae*

Glucose oxidase (ß-D:oxygen 1-oxidoreductase, EC 1.1.3.4) from *Aspergillus niger*
catalyses the oxidation of ß-D-glucose to glucono-δ-lactone and the concomitant
reduction of molecular oxygen to hydrogen peroxide. The fungal enzyme consists of a
homodimer of molecular weight 150,000 containing two tightly bound FAD co-factors.

Beside the use in glucose detection kits the enzyme is useful as a source of hydrogen peroxide in food preservation. The gene was cloned from both cDNA and genomic libraries, the single open reading frame contains no intervening sequences and encodes a protein of 605 amino acids (see reference 23).

5   With the help of two proper oligonucleotides the coding part of the sequence is adjusted in a one-step modifying procedure by PCR in such a way that a fusion gene product will be obtained coding for glucose oxidase and the C-terminal cell wall anchor of the *FLO1* gene product or α-agglutinin. Thus, some of the plasmids described in former EXAMPLES can be utilized to integrate the corresponding

10   sequence in-frame between one of the signal sequences used in the EXAMPLES and the *Nhe*I/*Hind*III part of the AGα1 gene.

Since dimerisation of the two monomers might be a prerequisite for activity, in an alternative approach the complete coding sequence for glucose oxidase without the GPI anchor can be expressed in *S. cerevisiae* transformant which already contains the

15   fusion construct. This can be fulfilled by constitutive expression of the fusion construct containing the GPI anchor with the help of the *GAPDH* or *PGK* promoter for example. The unbound not-anchored monomer can be produced by using a DNA construct comprising an inducible promoter, as for instance the *GAL7* promoter.

20

EXAMPLE 7   Process to convert raffinose, stachyose and similar sugars in soy extracts with α-galactosidase/α-agglutinin immobilized on yeasts

The yeast transformed with plasmid pUR2969 can be cultivated on large scale. At regular intervals during cultivation the washed cells should be analyzed on the

25   presence of α-galactosidase activity on their surface with methods described in EXAMPLE 1. When both cell density and α-galactosidase activity/biomass reach their maximum, the yeast cells can then be collected by centrifugation and washed. The washed cells can then be added to soy extracts. The final concentration of the yeast cells can vary between 0.1 and 10 g/l, preferably the concentration should be

30   above 1 g/l. The temperature of the soy extract should be < 8 °C to reduce the metabolic activity of the yeast cells. The conversion of raffinose and stachyose can be analyzed with HPLC methods and after 95 % conversion of these sugars the yeasts

25

cells can be removed by centrifugation and their α-galactosidase activity/g biomass
can be measured. Centrifugates with a good activity can be used in a subsequent
conversion process, whereas centrifugates with an activity of less then 50 % of the
original activity can be resuscitated in the growth medium and the cells can be
5   allowed to recover for 2 to 4 hours. Thereafter the cells can be centrifuged, washed
and subsequently be used in a subsequent conversion process.

**EXAMPLE 8   Production of biosurfactants using *Humicola*
lipase/α-agglutinin immobilized on yeasts.**

10   The yeast transformed with plasmid pUR2972 or pUR2973 can be cultivated on large
scale. At regular intervals during cultivation the washed cells can be analyzed on the
presence of lipase activity on their surface with methods described in EXAMPLE 1.
When both cell density and lipase/biomass reache their maximum, the yeast cells can
be collected by centrifugation and washed. The washed cells can be suspended in a
15   small amount of water and added to a reactor tank containing a mix of fatty acids,
preferably of a chain length between 12-18 carbon atoms and sugars, preferably
glucose, galactose or sucrose. The total concentration of the water (excluding the
water in the yeast cells) might be below 0.1 %. The final concentration of the yeast
cells can vary between 0.1 and 10 g/l, preferably the concentration is above 1 g/l. The
20   tank has to be kept under an atmosphere of N_2 and CO_2 in order to avoid oxidation
of the (unsaturated) fatty acids and to minimize the metabolic activity of the yeasts.
The temperature of mixture in the tank should be between 30-60 °C, depending on
type of fatty acid used. The conversion of fatty acids can be analyzed with GLC
methods and after 95 % conversion of these fatty acids the yeasts cells can be
25   removed by centrifugation and their lipase activity/g biomass can be measured.
Centrifugates with a good activity can be used in a subsequent conversion process,
whereas centrifugates with an activity of less then 50 % of the original activity can be
resuscitated in the growth medium and the cells can be allowed to recover for 2 to 8
hours. Thereafter the cells can be centrifuged again, washed and used in a subsequent
30   conversion process.

EXAMPLE 9   Production of special types of triacylglycerols using *Rhizomucor miehei* lipase/α-agglutinin immobilized on yeasts.

The yeast transformed with plasmid pUR2981 or pUR2982 can be cultivated on a large scale. At regular intervals during cultivation the washed cells can be analyzed on

5   the presence of lipase activity on their surface with methods described in EXAMPLE 1. When both cell density and lipase/biomass reach their maximum, the yeast cells can be collected by centrifugation and washed. The washed cells can be suspended in a small amount of water and can be added to a reactor tank containing a mix of various triacylglycerols and fatty acids. The total concentration of the water (excluding

10 the water in the yeast cells) might be below 0.1 %. The final concentration of the yeast cells can vary between 0.1 and 10 g/l, preferably the concentration is above 1 g/l. The tank has to be kept under an atmosphere of N_2 and CO_2 in order to avoid oxidation of the (unsaturated) fatty acids and to minimize the metabolic activity of the yeasts. The temperature of mixture in the tank should be between 30-70 °C,

15 depending on types of triacylglycerol and fatty acid used. The degree of interesterification can be analyzed with GLC/MS methods and after formation of at least 80 % of the theoretical value of the desired type of triacylglycerol the yeasts cells can be removed by centrifugation and their lipase activity/g biomass can be measured. Centrifugates with a good activity can be used in a subsequent conversion process,

20 whereas centrifugates with an activity of less then 50 % of the original activity is resuscitated in the growth medium and the cells should be allowed to recover 2 to 8 hours. After that the cells can be centrifuged, washed and used in a subsequent interesterification process.

Baker's yeasts of strain MT302/1C, transformed with either plasmid pSY13 or

25 plasmid pUR2969 (described in EXAMPLE 1) were deposited under the Budapest Treaty at the Centraalbureau voor Schimmelcultures (CBS) on 3 July 1992 under provisional numbers 330.92 and 329.92, respectively.

EXAMPLE 10   Immobilized *Humicola* lipase/FLO1 fusion on the surface of *S.*
30   *cerevisiae*

Flocculation, defined as "the (reversible) aggregation of dispersed yeast cells into flocs" (see reference 24), is the most important feature of yeast strains in industrial

fermentations. Beside this it is of principal interest, because it is a property associated with cell wall proteins and it is a quantitative characteristic. One of the genes associated with the flocculation phenotype in *S. cerevisiae* is the *FLO1* gene. The gene is located at approximately 24 kb from the right end of chromosome I and the DNA

5   sequence of a clone containing major parts of *FLO1* gene has very recently been determined (see reference 26). The sequence is given in Figure 11 and SEQ ID NO: 21 and 22. The cloned fragment appeared to be approximately   2 kb shorter than the genomic copy as judged from Southern and Northern hybridizations, but encloses both ends of the *FLO1* gene. Analysis of the DNA sequence data indicates that the

10   putative protein contains at the N-terminus a hydrophobic region which confirms a signal sequence for secretion, a hydrophobic C-terminus that might function as a signal for the attachment of a GPI-anchor and many glycosylation sites, especially in the C-terminus, with 46,6 % serine and threonine in the arbitrarily defined C-terminus (aa 271-894). Hence, it is likely that the *FLO1* gene product is localized in an

15   orientated fashion in the yeast cell wall and may be directly involved in the process of interaction with neighbouring cells. The cloned *FLO1* sequence might therefore be suitable for the immobilization of proteins or peptides on the cell surface by a different type of cell wall anchor.

Recombinant DNA constructs can be obtained, for example by utilizing the DNA

20   coding for amino acids 271-894 of the *FLO1* gene product, i.e. polynucleotide 811-2682 of Figure 11. Through application of two PCR primers pcrflo1 (see SEQ ID NO: 23) and pcrflo2 (see SEQ ID NO: 26) *Nhe*I and *Hind*III sites can be introduced at both ends of the DNA fragment. In a second step, the 1.4 kb *Nhe*I/*Hind*III fragment present in pUR2972 (either A or B) containing the C-terminal part of

25   α-agglutinin can be replaced by the 1.9 kb DNA fragment coding for the C-terminal part of the FLO1 protein, resulting in plasmid pUR2990 (see Figure 12), comprising a DNA sequence encoding (a) the invertase signal sequence (*SUC2*) preceding (b) the fusion protein consisting of (b.1) the lipase of *Humicola* (see reference 16) followed by (b.2) the C-terminus of FLO1 protein (aa 271-894).

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PCR oligonucleotides for the in frame connection of the genes encoding the
Humicola lipase and the C-terminal part of the *FLO1* gene product.

```
                                    S   N   Y   A   V   S   T
primer pcrflo1      5'- GAATTC GCT AGC AAT TAT GCT GTC AGT ACC - 3'
                                NheI   ||| ||| ||| ||| ||| |||
FLO1 gene (non-coding strand) 3'- AGT TTA ATA CGA CAG TCA TGG TGA - 5'
(for the part of the non-coding strand see SEQ ID NO: 24)
```

```
FLO1 coding strand  5'-AATAA AATTCGCGTTCTTTTTACG - 3'
                          ||||||||||||||||||||||
primer pcrflo2:          3'-TTAAGCGCAAGAAAAATGC TTCGAACTCGAG - 5'
                                                  HindIII
(for the part of the coding strand see SEQ ID NO: 25)
```

Plasmid pUR2972 (either A or B) can be restricted with *Nhe*I (partial) and *Hind*III
and the *Nhe*I/*Hind*III fragment comprising the vector backbone and the lipase gene
can be ligated to the correspondingly digested PCR product of the plasmid containing
the *FLO1* sequence, resulting in plasmid pUR2990, containing the *GAL7* promoter,
the *S. cerevisiae* invertase signal sequence, the chimeric lipase/*FLO1* gene, the yeast 2
μm sequence, the defective *Leu2* promoter and the *Leu2* gene. This plasmid can be
transformed into *S. cerevisiae* and the transformed cells can be cultivated in YP
medium including galactose as inductor.

The expression, secretion, localization and activity of the chimeric lipase/FLO1
protein can be analyzed using similar procedures as given in Example 1.

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CLAIMS

1. A method for immobilizing an enzyme, comprising the use of recombinant DNA
   techniques for producing an enzyme or a functional part thereof linked to the cell
   wall of a host cell, preferably a microbial cell, and whereby the enzyme or
   functional fragment thereof is localized at the exterior of the cell wall.

2. The method of claim 1, wherein the enzyme or the functional part thereof is
   immobilized by linking to the C-terminal part of a protein that ensures anchoring
   in the cell wall.

3. A recombinant polynucleotide comprising a structural gene encoding a protein
   providing catalytic activity and at least a part of a gene encoding a protein
   capable of anchoring in a eukaryotic or prokaryotic cell wall, said part encoding
   at least the C-terminal part of said anchoring protein.

4. The polynucleotide of claim 3, further comprising a sequence encoding a signal
   peptide ensuring secretion of the expression product of the polynucleotide.

5. The polynucleotide of claim 4, wherein the signal peptide is derived from a
   protein selected from the group consisting of glycosyl-phosphatidyl-inositol (GPI)
   anchoring protein, α-factor, α-agglutinin, invertase or inulinase, α-amylase of
   Bacillus, and proteinases of lactic acid bacteria.

6. The polynucleotide of any of claims 3-5, wherein the protein capable of anchoring
   in the cell wall is selected from the group consisting of α-agglutinin, AGA1,
   FLO1, Major Cell Wall Protein of lower eukaryotes, and proteinases of lactic
   acid bacteria.

7. The polynucleotide of any of claims 3-6, operably linked to a promoter,
   preferably an inducible promoter.

8. The polynucleotide of any of claims 3-7, wherein the protein providing catalytic activity is a hydrolytic enzyme, e.g. a lipase.

9. The polynucleotide of any of claims 3-7, wherein the protein providing catalytic activity is an oxidoreductase, e.g. an oxidase.

10. A recombinant vector comprising a polynucleotide as claimed in any of claims 3-9.

11. The recombinant vector of claim 10, wherein the protein providing catalytic activity exhibits said catalytic activity when present in a multimeric form, said vector further comprising a second polynucleotide comprising a structural gene encoding the same protein providing catalytic activity combined with a sequence encoding a signal peptide ensuring secretion of the expression product of said second polynucleotide, said second polynucleotide being operably linked to a regulatable promoter, preferably an inducible or repressible promoter.

12. A chimeric protein encoded by a polynucleotide as claimed in any of claims 3-9.

13. A host cell, preferably a microorganism, containing a polynucleotide as claimed in any of claims 3-9 or a vector as claimed in claim 10 or 11.

14. A host cell, preferably a microorganism, containing a polynucleotide as claimed in any of claims 3-9 or a vector as claimed in claim 10, wherein the protein providing catalytic activity exhibits said catalytic activity when present in a multimeric form, said microorganism further comprising a second polynucleotide comprising a structural gene encoding the same protein providing catalytic activity combined with a sequence encoding a signal peptide ensuring secretion of the expression product of said second polynucleotide, said second polynucleotide being operably linked to a regulatable promoter, preferably an inducible or repressible promoter and said second polynucleotide being present either in another vector or in the chromosome of said microorganism.

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15. The host cell or microorganism of claim 13 or 14, having at least one of said polynucleotides integrated in its chromosome.

16. A host cell, preferably a microorganism, having a protein as claimed in claim 12 immobilized on its cell wall.

17. The host cell or microorganism of any of claims 13-16, which is a lower eukaryote, in particular a yeast.

18. A process for carrying out an enzymatic process by using an immobilized catalytically active protein, wherein a substrate for said catalytically active protein is contacted with a host cell or microorganism as claimed in any of claims 13-17.

* * * * *